Detection of antizona pellucida antibodies in the sera from premature ovarian failure patients by a highly specific test

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Objective: To develop a highly specific test for the detection of antizona pellucida (ZP) antibodies in the sera from premature ovarian failure (POF) patients.
Design: Laboratory study.
Setting: University hospital.
Patient(s): Twenty-seven idiopathic POF patients, 30 control women, and 30 healthy males.
Intervention(s): Anti-ZP antibodies were detected by the microdot assay using a very small amount of human ZP or porcine ZP. The effect of anti-ZP antibodies on sperm-ZP binding was examined by hemizona assay.
Main outcome measure(s): Results from the microdot assay and hemizona assay.
Result(s): By the microdot assay using human ZP, the sera from POF patients reacted significantly stronger than those of control women and healthy males. However, no obvious difference could be found by the same assay using porcine ZP among these three groups. Anti-ZP antibodies against sera from some POF patients showed significant blocking effects on sperm-ZP binding assessed by hemizona assay. Anti-ZP antibodies were detected in 7 of 27 POF patients, while none were detected in control women and healthy males.
Conclusion(s): Some idiopathic POF patients have anti-ZP antibodies in their sera, which were detected with high specificity by a newly developed microdot assay using a very small amount of human ZP. (Fertil Steril® 2007;88:925–32. ©2007 by American Society for Reproductive Medicine.)

Key Words: Antizona pellucida (ZP) antibody, human ZP, premature ovarian failure, sperm-ZP binding

Premature ovarian failure (POF) is a syndrome characterized by development of amenorrhea before the age of 40 years with elevated serum gonadotropin levels and low serum estrogen levels (1). It occurs in 1% of women, in 10%–28% of women with primary amenorrhea, and in 4%–18% of those with secondary amenorrhea (2, 3).

The etiology of POF is thought to involve a wide spectrum of pathogenic mechanisms including chromosomal, genetic, environmental (radiation or medicine), metabolic, and autoimmune factors (4). In the past 3 decades, some investigators have reported the clinical association of POF with other autoimmune disorders. Approximately 20%–40% of patients with POF have associated autoimmune disorders (4–7). In a large proportion of cases no cause has been identified, and these cases are classified as idiopathic POF. Autoimmune mechanisms are involved in the pathogenesis of up to 30% of cases in idiopathic POF (8, 9).

The presence of organ-specific autoimmune antibodies may support the role of autoimmune mechanisms in endocrine diseases. For example, antiovarian antibodies in patients with POF were detected by different methods, the most common being indirect immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA). Numerous investigators have tested POF patient groups using IF, ELISA, and other methods. The prevalence of antiovarian antibodies ranges widely, between 2.2% and 69% of patients (10–16). Considering these variable results, the pathogenic roles of antiovarian antibodies in POF are still uncertain in terms of their specificity. Such conflicting results could be explained by the methodologic differences, relatively small number of patients, the different stages of the disease when tested, the differences of antigen sources, and the multiplicity of potential immune targets that comprised various steroidogenic enzymes, gonadotropins, and their receptors: the corpus luteum, oocyte, and zona pellucida (ZP) (9, 17).

Zona pellucida consists of glycoproteins that have strong antigenic potency. Therefore, anti-ZP antibodies may be one of the causes of autoimmune POF. Evidence showing that antibodies directed to ZP could cause POF has been demonstrated. Ovarian failure could be induced in rabbits immunized with porcine ZP proteins (18). Ovarian autoimmune disease was induced in B6AF1 mice by 15-amino acid peptides from mouse ZP3 (19). Because ZP proteins are conserved among mammals (mouse and human ZP3 proteins are 67% identical), those animal models may lead to a better understanding of the pathogenesis of human autoimmune oophoritis. Circulating antibodies against ZP were consistently detected by immunohistochemical assay in mice with autoimmune oophoritis that could be induced with high incidence by thymectomy at 3 days of age and caused great oocyte loss

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(20, 21). Smith and Hosid (22) reported two cases of POF associated with antibodies directed against ZP .

After antigenic crossreactivity between human and porcine ZP was revealed (23, 24), porcine ovaries and oocytes have been generally used to detect anti-ZP antibodies because they are easy to obtain in large quantities. Anti-ZP antibodies have also been discussed as a possible cause of infertility in women for the blocking effect on sperm-ZP binding. Some studies reported that anti-ZP antibodies were detected with high incidence in infertile women by IF using porcine oocytes and ZP (24–26). However, the specificity of immune reaction with porcine oocytes has been questioned because of the nonspecific reaction of porcine ZP. To have better specificity in the detection of anti-ZP antibodies using porcine ZP, passive hemagglutination reaction or absorptive treatment techniques were developed (27–29). Although such passive hemagglutination reaction or absorption techniques contributed to better specificity and reliability for detecting anti-ZP antibodies, the significance of these antibodies remains unclear because of possible nonspecific reactions.

Therefore, we concluded that it is necessary to develop a highly specific test for the detection of anti-ZP antibodies using human ZP. However, one limitation is that it is still difficult to obtain human ZP in large quantities, even in the era of modern assisted reproductive technology. In the present study, a microdot assay using a very small amount of human ZP was developed. Moreover, sera containing anti-ZP antibodies from patients with idiopathic POF patients were used to investigate the blocking effect on sperm-ZP binding.

MATERIALS AND METHODS

Approval for the study was obtained from the Institutional Ethics Committee of Jichi Medical University Hospital, and informed consent was obtained from all patients.

Serum Samples

Serum samples were collected from 27 women diagnosed with idiopathic POF. Criteria for POF included secondary amenorrhea, age <40 years at the onset of ovarian failure, persistent high serum gonadotropin levels, and low serum estrogen levels (1). In addition, to classify as idiopathic POF, patients with abnormal karyotype, previous pelvic irradiation, operative castration, and previous cytotoxic chemotherapy were excluded, and patients with no cause identified were selected (8).

As the control, sera from 30 fertile females without disorder on ovulation and fertilization were obtained. Women with regular ovulatory cycle and experience of conception, who had undergone at least two cycles of IVF treatment with total number of oocytes retrieved >10 and 100% fertilization rate in their IVF cycles, were defined as the control women. Sera from 30 normally healthy volunteer males were also obtained as the negative control. Because anti-ZP antibodies were autoimmune antibodies, there was a possibility that normal cycling women had these antibodies. On the other hand, healthy males were not expected to have these antibodies logically and suitable for the true negative control.

Whole serum was diluted 1:4 in phosphate-buffered saline (PBS) without calcium containing 3% bovine serum albumin as the test serum sample.

Preparation of Human and Porcine ZP

Under informed consent, human oocytes that failed to fertilize in vitro or that were not used because of being immature were obtained from infertile couples that had undergone assisted reproductive technology. They were stored until use at 4°C in a solution of 0.5 M ammonium sulfate with 1 M magnesium chloride and 0.1% dextran. After hundreds of human oocytes were stored, they were put to use.

Porcine ovaries obtained from a local slaughterhouse were frozen and stored. The porcine oocytes were collected from thawed porcine ovaries by aspiration using syringes and needles. Several sets of ten oocytes from one ovary could be collected at one time.

Human and porcine ZP were individually separated from their cytoplasm mechanically by pipetting with a narrow glass pipette with a caliber smaller than the oocyte diameter. They were then placed in PBS (100 μg/mL = 100 ZP/30 μL PBS) at 75°C for 30 minutes.

Microdot Assay

The microdot assay is an immunostaining method, and anti-ZP antibodies in the sera can be detected when combined with soluble ZP proteins used for antigen.

A small piece of nitrocellulose membrane measuring 1.5 cm in width and 1.0 cm in length was divided into six areas with two vertical lines and one horizontal line. On each of the upper panels, a microdot was made with serial dilution ($\times$1, $\times$3, $\times$9) of 0.2 μL soluble human ZP, while on that of the lower panels, it was made with soluble porcine ZP. Approximately 0.7 ZP, 0.2 ZP, and 0.07 ZP were contained in one dot of 0.2 μL soluble human and porcine ZP with serial dilution $\times$1, $\times$3, and $\times$9, respectively. After drying, they were blocked in PBS containing 3% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 10 minutes. As a primary antibody reaction, 180 μL of the test serum was added on the nitrocellulose membrane. After incubation of the nitrocellulose membranes with patients' sera in a moist chamber at 4°C overnight, they were washed three times in PBS containing 0.02% Tween 20 (Kanto Chemical Co., Inc., Tokyo, Japan, 40350-32, polyoxyethylenesorbitan monolaurate, nonionic detergent) for 5 minutes. The excess PBS containing 0.02% Tween 20 was aspirated off, then the nitrocellulose membranes were incubated in horseradish peroxidase-conjugated antihuman IgG (Sigma Chemical Co.) diluted 1:1,000 in PBS as a second reaction at room temperature for 1 hour. Then, they were washed three times in PBS containing 0.005% Tween 20 for 5 minutes. The dots on the nitrocellulose membranes were colored and visualized by chloronaphthol, 3 mg of which was dissolved in 1 mL of methanol with 5 mL of PBS and 2 μL of H_2O_2 (Kanto

Chemical Co., Inc.). The nitrocellulose membranes were washed in distilled water and dried.

Analysis

The color development of each dot was evaluated by computer-assisted image analysis, and expressed the existence of anti-ZP antibody in the sera. After scanning the nitrocellulose membranes using a flat bed scanner in 256 grayscales, the density of staining was measured by NIH image (a public domain image processing and analysis program for the Macintosh, developed at the National Institutes of Health [NIH], USA). NIH image number (NIN) was calculated by subtracting the density of the background stain from that of the dot stain. All serum samples were assayed, and then the NIN of each dot was calculated and compared.

Hemizona Assay

The hemizona assay (HZA) was developed originally to predict the fertilizing potential of spermatozoa (30). The effect of anti-ZP antibodies in the sera from POF patients on sperm-ZP interaction was investigated by the HZA as follows. A pair of hemizona (HZ) was made of one oocyte cut into halves by hand using biocut blade (Feather Safety Razor Co., Ltd., Osaka, Japan), a microblade for biotechnology bisection, on a phase contrast microscope. One HZ was placed in a 50 μL drop of each test serum from a POF patient under mineral oil for 1 hour at 37°C in 5% CO_2 in air, while the other matched HZ was placed in a drop of the serum from a healthy male selected as a control. After incubation, each HZ was washed five times in the culture medium and inseminated by swim-up sperm obtained from proven fertile healthy males. Semen was centrifuged and washed twice in sperm-washing medium after liquefaction. The motile spermatozoa were collected by a standard swim-up technique and resuspended in the culture medium. Each preincubated HZ was exposed to 50 μL of sperm suspension (2.5×10^5/mL) and coincubated for 1 hour at 37°C in 5% CO_2 in air. After insemination, each HZ was removed and rinsed vigorously to detach loosely associated spermatozoa. The number of spermatozoa tightly bound to the outer HZ surface was counted.

The hemizona index (HZI) was calculated as follows: (the number of spermatozoa bound to HZ preincubated with POF patient's serum) divided by (the number of spermatozoa bound to HZ preincubated with control serum) × 100. Sera from POF patients that blocked sperm-ZP tight binding at least 50% (HZI <50) using HZA were considered to have a blocking effect on fertilization (31). Those sera were also considered to have the anti-ZP antibodies.

Sera from representative 10 of 27 POF patients were selected for sampling and used for the analysis by HZA.

Statistical Methods

Student's *t* test and chi-square test were used for data analysis, and $P<.05$ was considered statistically significant. Statistical analysis was performed with Excel statistical software for Macintosh.

RESULTS

Characteristics of Idiopathic POF Patients

The characteristics of 27 idiopathic POF patients were as follows. In the POF patients, serum FSH levels were 95.6 ± 38.0 mIU/mL, and 10 of them showed serum FSH levels >100 mIU/mL. On the other hand, serum estradiol levels were as low as those in postmenopausal women. Notably, 19 patients had serum estradiol levels under the lowest limit of examination (<10.0 pg/mL). The age at the onset of amenorrhea of the POF patients was 23–38 years old (mean 30.0 years).

Other autoimmune antibodies: antinuclear antibodies, lupus anticoagulant antibodies, antithyroid antibodies, anti-double-stranded DNA antibodies, rheumatoid factors, and anticardiolipin and anti-beta2-glycoprotein-1 antibodies, were also examined (data not shown). There was no relation among these autoimmune antibodies. In final, about these autoimmune antibodies, there was no characteristic in the POF patients with anti-ZP antibodies compared with others without anti-ZP antibodies.

Results of the Microdot Assay

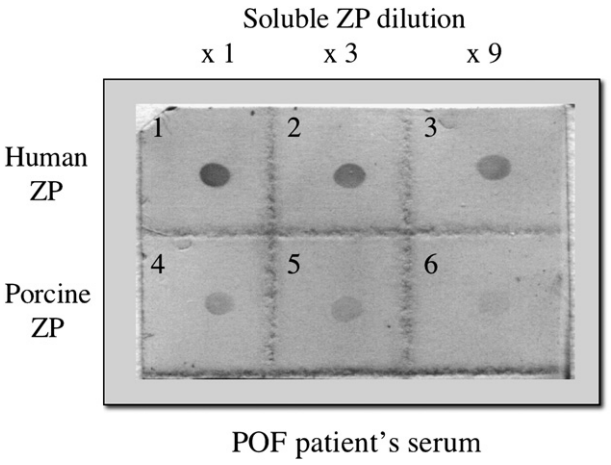
The nitrocellulose membrane after the microdot assay of the serum from an idiopathic POF patient is shown in Figure 1. The dots on the upper panels demonstrated reaction to soluble human ZP, and those on the lower panels demonstrated reaction to soluble porcine ZP. The density of dots were stained dose dependently in both panels.

NIN after the microdot assay using soluble human ZP and soluble porcine ZP are presented in Figure 2. The NIN (14.0 ± 14.2; mean ± SD) of the sera from POF patients was significantly higher ($P<.01$) than both that (5.6 ± 5.9) of control women and that (0.2 ± 1.4) of healthy males by the microdot assay using soluble human ZP. There was also significant difference ($P<.01$) between the NIN of control women and that of healthy males (Fig. 2A). In contrast, there was no significant difference among the NIN (36.2 ± 23.9) of POF patients, that (23.9 ± 21.1) of control women, and that (25.8 ± 22.1) of healthy males by the microdot assay using soluble porcine ZP (Fig. 2B). These results represent that the microdot assay using soluble human ZP is superior in specificity to the assay using soluble porcine ZP to detect anti-ZP antibodies in the sera.

NIN of the sera from POF patients, control women, and healthy males by the microdot assay using soluble human ZP were compared (Fig. 3). None of the sera from healthy males reacted with soluble human ZP except one with very low NIN (7.4). Sera from 12 of 30 (40%) control women did not react (NIN = 0); all the other 18 sera reacted with low NIN (<18), and the highest NIN of them was 17.0. Fewer (7 of 27, 26%) sera from POF patients did not react, while seven other POF patients strongly reacted with high NIN (>18). The NIN of control women was 5.6 ± 5.9 (mean ± SD) and the NIN of mean +2SD of them was 17.5. Therefore, the sera showed their NIN above 17.5 by the microdot assay

FIGURE 1

NC membrane after microdot assay. Microdots were made with a serial dilution (×1, ×3, ×9) of 0.2 μL soluble human ZP on each area of the upper panels of the nitrocellulose membrane (1–3), while they were made with soluble porcine ZP on that of the lower panels (4–6). Approximately 0.7 ZP, 0.2 ZP, and 0.07 ZP were contained in one dot of 0.2 μL soluble human and porcine ZP with a serial dilution of ×1, ×3, and ×9, respectively. This figure was reacted with the serum from a POF patient. The density of dots were stained dose dependently in both panels.



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using soluble human ZP would be decided as positive for the microdot assay.

Results of HZA and Criterion of Value Point of NIN

All HZI and NIN of sera from representative 10 (Fig. 3; ○ and ●) of 27 POF patients are presented in Table 1.

For example, the sera from a POF patient (Table 1; code 5593017) with anti-ZP antibodies demonstrated a blocking effect on sperm-ZP tight binding and the test result is shown in Figure 4. The number of spermatozoa bound to the HZ (A) treated with the patient's serum and HZ (B) treated with the control serum were 49 and 104, respectively. The HZI (hemizona index) was calculated as follows: $49/104 \times 100 = 47.1$.

The value point of NIN that presented anti-ZP antibodies positive was defined as follows. Sera from three (Fig. 3; ○) of the representative 10 patients inhibited sperm-ZP tight binding (HZI <50), and they were considered to have anti-ZP antibodies. The other seven patients (Fig. 3; ●) had HZI >50, and they were not considered to have anti-ZP antibodies. The NIN of the sera from these three POF patients was 39.6 ± 26.4, while that of the other seven POF patients was 8.8 ± 6.7. There was a significant difference in NIN between the two groups (P=.01) (Fig. 5). The lowest NIN of these three POF patients considered to have anti-ZP antibodies was 18.3, and the highest NIN of these seven POF patients considered not to have anti-ZP antibodies was 16.9. On the other hand, as mentioned above, the NIN of control women was 5.6 ± 5.9 (mean ± SD) (0∼17.0), and the NIN of mean +2SD was 17.5. Based on these results of the microdot assay and HZA, the sera from POF patients, which showed their NIN above 18 by the microdot assay using soluble human ZP, were decided as positive for anti-ZP antibodies, which had obvious blocking effects on sperm-ZP binding. Therefore, the other 4 of 17 sera (Fig. 3; ◇ and ◆), that showed their NIN above 18 by the microdot assay, were considered as anti-ZP antibodies positive according to the established criteria. Taken together, 7 of 27 POF patients were considered to be anti-ZP antibodies positive (Fig. 3; ○ and ◇).

DISCUSSION

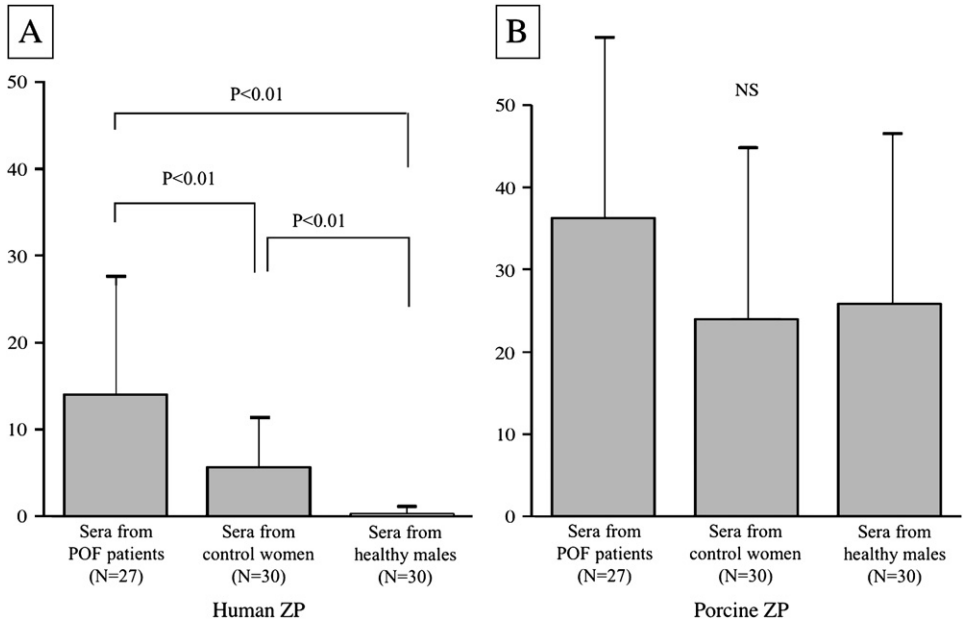
Numerous studies have reported that autoimmunity was associated with POF, especially with idiopathic POF (4–16). Antiovarian antibodies were considered one of the causes of idiopathic POF, and they were detected using variable methods (5–14). Although the pathology and mechanism of antiovarian antibodies on POF is still unclear, it is likely that autoimmunity and these antibodies contribute to the etiology of idiopathic POF. In those antiovarian antibodies, anti-ZP antibodies most likely cause POF, and some mammal experimental models support this hypothesis (18–20). Some studies have reported the existence of antibodies directed against ZP in human POF patients (22, 32). Being associated with autoimmune disease, POF patients might have productive ability of any autoimmune antibodies. Anti-ZP antibodies might also be one of the autoimmune antibodies that were produced by POF patients. We suppose anti-ZP antibodies are mainly the cause, and also might be the result of POF.

However, to detect anti-ZP antibodies, a commercial kit was used with monkey ovaries and porcine ZP, not human ZP as the antigen. Questions of reliability still remained. Because the microdot assay that we developed used human ZP as the antigen, the anti-ZP antibodies detected by microdot assay in the sera from idiopathic POF patients were reliable. It is certain that anti-ZP antibodies are related to the cause and result of POF.

In some infertile women, anti-ZP antibodies are also considered to take part in infertility because of their blocking effects on sperm-ZP binding. Zona pellucida is composed of some biochemical distinct proteins and surrounds the mammalian oocyte. Zona pellucida proteins are considered to act as the sperm receptor in fertilization. Therefore, anti-ZP antibodies are considered to act as the inhibitor to the receptor. Monoclonal antibodies to a glycoprotein family of porcine ZP showed a significant blocking effect on human sperm binding and penetration of human ZP (33). Antibodies against bonnet monkey ZP proteins significantly inhibited the binding of human spermatozoa to ZP in HZA (34).

FIGURE 2

Comparison of NIN of POF patients, control women, and healthy males by the microdot assay using soluble human and porcine ZP. The NIN (NIH image number: 14.0 ± 14.2; mean ± SD) of the sera from POF patients was significantly higher ($P<.01$) than both that (5.6 ± 5.9) of control women and that (0.2 ± 1.4) of healthy males by the microdot assay using soluble human ZP. There was also a significant difference between the NIN of control women and that of healthy males (**A**). In contrast, there was no significant difference among the NIN (36.2 ± 23.9) of POF patients, that (23.9 ± 21.1) of control women, and that (25.8 ± 22.1) of healthy males by the microdot assay using soluble porcine ZP (**B**). These results indicate that the microdot assay using human ZP is specific, while that using porcine ZP is not specific.



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Preexposure of human ZP to the sera showing positive IF greatly diminished the number of spermatozoa of normal quality that bound to and penetrated across human ZP (28). In our study, sera from some idiopathic POF patients with anti-ZP antibodies detected by the microdot assay using soluble human ZP showed strong blocking effects on sperm-ZP binding by the HZA (Table 1). These findings suggest that human anti-ZP antibodies also had inhibitory effects on sperm-ZP binding, and might be one of causes of infertility.

Papale et al. (35) showed low fertilization rates in patients with anti-ZP antibodies in IVF treatment cycles. Mardesic et al. (36) reported that fertilization failure after standard IVF occurred in most cases with anti-ZP antibodies, and intracytoplasmic sperm injection was the method of choice in anti-ZP antibody-positive infertile couples. Although they detected anti-ZP antibodies by the hemagglutination test, the passive hemagglutination test, or ELISA, as far as they used porcine antigenic fractions and porcine ZP as the antigen, their results likely had problems in reliability and accuracy. In our study, anti-ZP antibodies were detected in the sera from idiopathic POF patients, control women, and healthy males by the microdot assay using both soluble human and porcine ZP. No obvious difference could be found among the three groups in their reactions to porcine ZP. There was no difference among their NIN by the microdot assay using soluble porcine ZP (Fig. 2B). However, by the microdot assay using soluble human ZP, the NIN of sera from idiopathic POF patients was significantly higher than that of control women and that of healthy males (Fig. 2A). No sera from healthy males reacted (NIN = 0) to human ZP proteins except only one with very low NIN (7.4) (Fig. 3). Sera from 12 of 30 (40%) control women did not react (NIN = 0); all the other 18 sera reacted with low NIN (<18). Fewer (7 of 27, 26%) sera from POF patients did not react, while seven other POF patients strongly reacted with high NIN (>18). These results clearly indicate that the microdot assay using soluble human ZP is specific, while that using soluble porcine ZP is not specific for the detection of anti-ZP antibodies. The scientific basis of the specificity of reactivity to human ZP and the nonspecificity of reactivity to porcine ZP is not certain yet. It would be expected to be clear by further molecule biological studies in the future.

Zona pellucida is an extracellular matrix surrounding the mammalian oocyte, and it plays an important role in attachment of sperm, fertilization, inhibition of polyspermic fertilization, and protection of early embryos. It is very important

FIGURE 3

NIN of POF patients, control women, and healthy males and the value point of NIN by the microdot assay using soluble human ZP. NIN (NIH image number) of the sera from POF patients (○, ●, ◇, ◆), control women (■), and healthy males (▲) by the microdot assay using soluble human ZP were compared. None of the sera from healthy males reacted with soluble human ZP, except only one with very low NIN (7.4). Sera from 12 of 30 control women did not react (NIN = 0); the other 18 sera reacted with low NIN (<18), and the highest NIN was 17.0. The NIN of control women was 5.6 ± 5.9 (mean ± SD), and the NIN of mean +2SD was 17.5. There were fewer (7 of 27) sera from POF patients who did not react, while some other POF patients strongly reacted with high NIN (>18). Hemizona assay was performed using sera from a representative 10 (○ and ●) of 27 POF patients, and the HZI was calculated. Sera from 3 (○; NIN: 69.1, 31.3, 18.3) of a representative 10 patients inhibited sperm-ZP tight binding (HZI <50), and they were considered to have anti-ZP antibodies. The other seven patients (●; NIN: 16.9, 15.5, 10.6, 10.1, 8.7, 0, 0) had HZI >50, and they were not considered to have anti-ZP antibodies. The lowest NIN of these three POF patients was 18.3, and the highest NIN of these seven POF patients was 16.9. According to these results, the value point of NIN was defined as 18.0, represented by the dotted line in the figure. The sera from POF patients who showed their NIN above 18 by the microdot assay using soluble human ZP were decided as positive for anti-ZP antibodies, which had obvious blocking effects on sperm-ZP binding. The other 17 POF patients (◇ and ◆) were divided into two groups of anti-ZP antibodies positive (◇) and negative (◆) according to the established criteria.

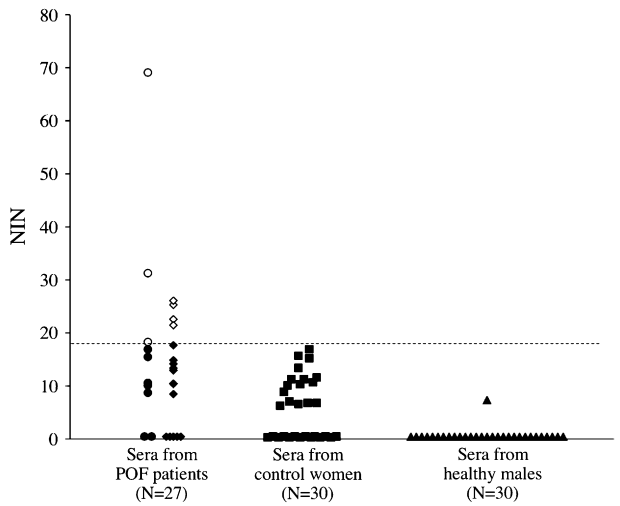

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TABLE 1

HZI and NIN of the sera from 10 POF patients.

Patient code	HZI[a]	NIN[b]
With inhibitory effect on zona binding[c]		
6060420	35.7	31.3
6160428	42.9	18.3
5593017	47.1	69.1
Without inhibitory effect on zona binding[d]		
6290068	60.0	10.1
6081461	70.3	10.6
6304398	71.9	0
3472255	77.5	16.9
0008411	81.3	8.7
6057871	94.1	0
5547997	105.0	15.5

Note: POF = premature ovarian failure.
[a] HZI: hemizona index.
[b] NIN: NIH image number.
[c] HZI < 50.
[d] HZI ≥ 50.

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to investigate anti-ZP antibodies that might have inhibitory effects on these ZP functions. All the past reported studies included great methodologic heterogeneity, such as the type of antiovarian or anti-ZP antibodies, the timing of test and blood sample collection, the selection of control groups, and so on. Therefore, no definitive conclusions regarding the incidence and the role of anti-ZP antibodies in POF and infertile women could have been drawn yet. Such indefinite and conflicting results are attributed to the use of porcine or other mammalian ZP for antigens, involving problems with specificity, instead of human ZP. To resolve these problems, it is necessary to develop a new method for the detection of anti-ZP antibodies using human ZP as the real antigen. As human oocytes or ZP are difficult to obtain in a large quantity, we developed a microdot assay that uses a very small amount of ZP. In this microdot assay, approximately 1.5 serum samples per one ZP could be tested on calculation. Less soluble ZP was used to make the microdots, more serum samples could be tested. By using this method, large amounts of homogenous ZP antigen was available, and soluble ZP antigen in equal condition was supplied to the microdot assay whenever anti-ZP antibodies were detected.

Some idiopathic POF patients demonstrated strong reactions to human ZP by the microdot assay, suggesting that the anti-ZP antibodies detected by the microdot assay might be a cause of POF. There was no difference in age, serum LH, and FSH levels between the two groups of idiopathic POF patients with or without anti-ZP antibodies. There was no correlation between other autoimmune antibodies and anti-ZP antibodies (data not shown). It is impossible to predict whether anti-ZP antibodies are positive or not in the sera from idiopathic POF patients by their characteristics.

FIGURE 4

A pair of HZ after HZA using the sera from POF patients with anti-ZP antibodies positive. A pair of HZ after HZA that demonstrated a blocking effect of serum from a POF patient (Table 1, code 5593017) with anti-ZP antibodies on sperm-ZP tight binding is shown. Before HZA, HZ (**A**) was preincubated with the serum from a POF patient (Table 1, code 5593017) with high NIN and was considered to be anti-ZP antibodies positive. The other matched HZ (**B**) was preincubated with control serum from one healthy male selected as being anti-ZP antibodies negative. There were fewer spermatozoa bound to HZ (**A**) preincubated with the sera from the POF patient with anti-ZP antibodies than HZ (**B**) preincubated with control serum. The number of spermatozoa bound to HZ (**A**) and HZ (**B**) were 49 and 104, respectively. The HZI was calculated as follows: $49/104 \times 100 = 47.1$.

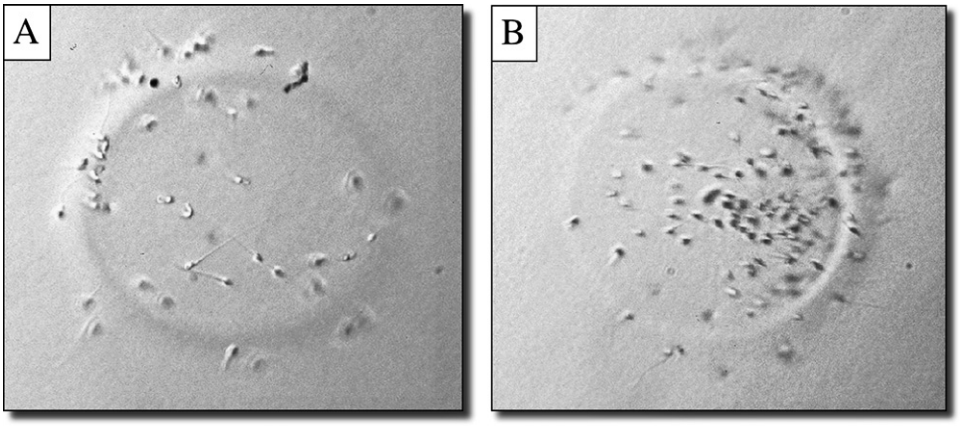

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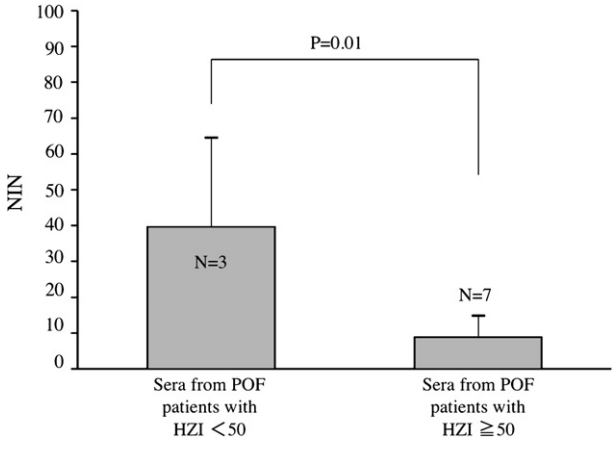
FIGURE 5

Comparison of NIN of POF patients with HZI <50 and with HZI ≥ 50. The NIN (NIH image number) of the sera from three POF patients with HZI <50 was 39.6 ± 26.4 (mean $\pm$ SD), while that of the other seven POF patients with HZI ≥ 50 was 8.8 ± 6.7 (mean $\pm$ SD). There was a significant difference of NIN between the two groups ($P=.01$).



Takamizawa. Detection of anti-ZP antibodies in POF patients. Fertil Steril 2007.

There were some idiopathic POF patients whose sera showed increased NIN by the microdot assay using soluble human ZP and strong blocking effect on sperm-ZP binding by HZA simultaneously (Fig. 5). These idiopathic POF patients were considered to have anti-ZP antibodies in their sera. The antibodies could be detected specifically by the microdot assay using soluble human ZP, and might be one of the causes of infertility with blocking effects on sperm-ZP binding. The microdot assay using soluble human ZP seems to be useful for detecting anti-ZP antibodies that are associated not only with the cause of idiopathic POF but also with sperm-ZP interaction. Further studies are required to show whether the microdot assay using soluble human ZP is useful for indicating that some unexplained infertility in women with a history of fertilization failure in IVF treatment cycles might have anti-ZP antibodies.

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